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Protein Knockdown Using Methyl Bestatin−Ligand Hybrid Molecules: Design and Synthesis of Inducers of Ubiquitination-Mediated Degradation of Cellular Retinoic Acid-Binding Proteins

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Abstract: Induction of selective degradation of target proteins by small molecules (protein knockdown) would be useful for biological research and treatment of various diseases. To achieve protein knockdown, we utilized the ubiquitin ligase activity of cellular inhibitor of apoptosis protein 1 (cIAP1), which is activated by methyl bestatin (MeBS, **2**). We speculated that formation of an artificial (nonphysiological) complex of cIAP1 and a target protein would be induced by a hybrid molecule consisting of MeBS (**2**) linked to a ligand of the target protein, and this would lead to cIAP1-mediated ubiquitination and subsequent proteasomal degradation of the target protein. To verify this hypothesis, we focused on cellular retinoic acid-binding proteins (CRABP-I and -II) and designed hybrid molecules (compounds **4**) consisting of MeBS (**2**) coupled via spacers of various lengths to *all-trans* retinoic acid (ATRA, **3**), a ligand of CRABPs. Compounds **4** induced selective loss of CRABP-I and -II proteins in cells. We confirmed that **4b** induced formation of a complex of cIAP1 and CRABP-II *in vitro* and induced proteasomal degradation of CRABP-II in cells. When neuroblastoma IMR-32 cells were treated with **4b**, the level of CRABP-II was reduced and cell migration was inhibited, suggesting potential value of CRABP-II-targeting therapy for controlling tumor metastasis. Our results indicate that **4b** possesses sufficient activity, permeability, and stability in cells to be employed in cellular assays. Hybrid molecules such as **4** should be useful not only as chemical tools for studying the biological/physiological functions of CRABPs but also as candidate therapeutic agents targeting CRABPs.

Introduction

Physiological degradation of proteins via the ubiquitin−proteasome system is crucial for regulating cellular functions, including the cell cycle, immunoresponses, and signal transduction.[1] In general, protein ubiquitination or polyubiquitination is mediated by sequential reactions of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Polyubiquitinated proteins are recognized and degraded by proteasome.[2] Many ubiquitin ligases (E3) have been reported, and it is thought that different E3 ligases have different specificities; i.e., they distinguish various proteins which are to be ubiquitinated.[3] A method for simply and selectively inducing posttranslational degradation of target proteins by regulating this system, which we call protein knockdown, would be useful for biological studies and medical research. It might also provide a new therapeutic strategy in cases where expression of target proteins is closely related to specific diseases.

Genetic techniques such as gene knockout and gene knockdown have been widely used for ablating target proteins and have been useful to uncover the biological functions of numerous proteins in cells or animals. However, complicated and time-consuming genetic manipulation is required for gene knockout. Gene knockdown using RNA interference is an easy method but cannot remove existing proteins and so is especially ineffective in the case of proteins with a long half-life. Therefore, other techniques which can rapidly remove or down-regulate proteins post-translationally would be desirable. One such technique is application of proteolysis-targeting chimeric molecules (protacs)[4] based on peptide structure.[5] Protacs have been reported to degrade target proteins. However, protacs possess peptide structure and must be polyargininated to endow them with sufficient membrane permeability for use in cellular systems.[6] Moreover, stability issues associated with their high molecular weight and vulnerable peptide bonds limit their broad

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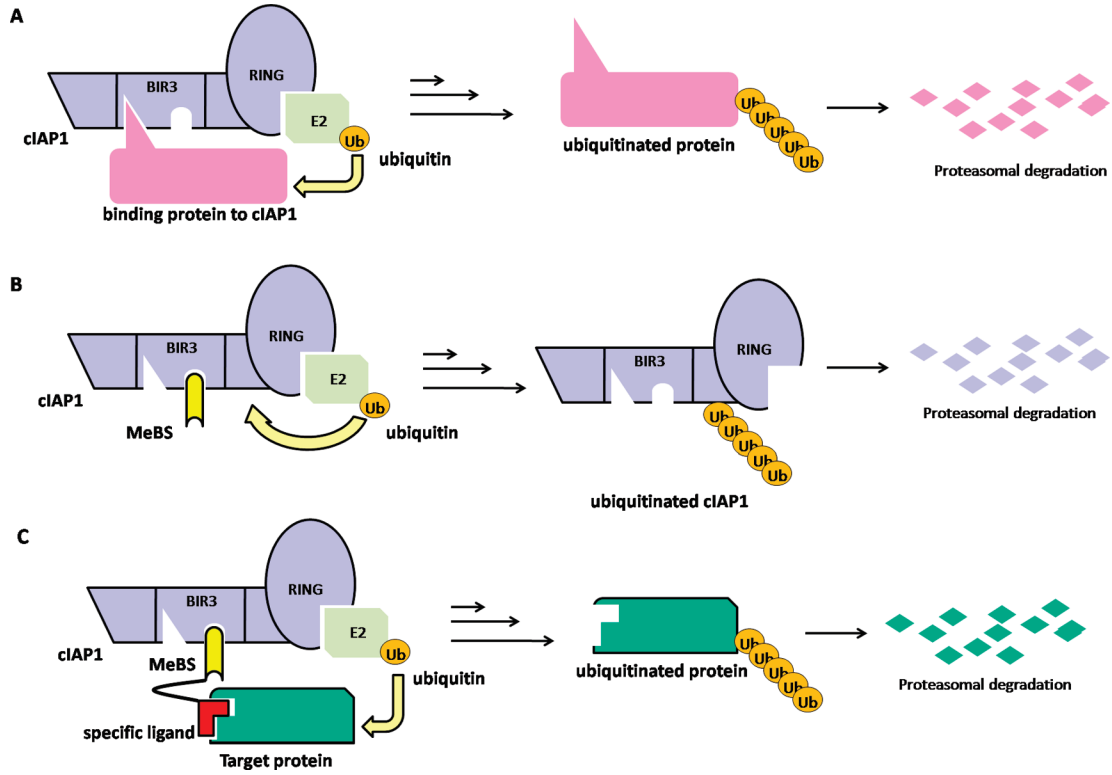
Scheme 1. (a) cIAP1 Induces Degradation of Binding Proteins. (b) Auto-Ubiquitination and Degradation of cIAP1. (c) Protein Knockdown Strategy



applicability, and other general methods to remove target proteins post-translationally in cells and animals would be desirable. Therefore, we attempted to develop a new approach, which we call protein knockdown, using small molecules to induce selective degradation of target proteins post-translationally.

To develop such a protein knockdown approach, we focused on cellular inhibitor of apoptosis protein 1 (cIAP1), which is one of the inhibitor of apoptosis proteins (IAPs) and is overexpressed in certain tumor cells.[7] It inhibits apoptosis induced by a variety of stimuli.[8] cIAP1 contains (i) three baculoviral IAP repeat (BIR) domains which interact with its binding proteins, including caspases, and (ii) one really interesting new gene (RING) finger domain involved in ubiquitin ligase activity.[7−9] cIAP1 promotes ubiquitination and proteasomal degradation of its binding proteins (Scheme 1a).[9] Furthermore, Naito's group reported that a class of bestatin ester analogues represented by methyl bestatin (MeBS, **2**) bind to the BIR3 domain of cIAP1 and promote autoubiquitination and degradation of cIAP1 (Scheme 1b).[10] Based on these observations, we hypothesized that a hybrid molecule consisting of MeBS (**2**) coupled to a ligand for a target protein might be able to induce cIAP1-mediated ubiquitination and proteasomal degradation of the target protein.

For a proof-of-concept study, we chose cellular retinoic acid binding proteins (CRABP-I and -II) as target proteins. These proteins reside in cytoplasm and specifically bind to *all-trans* retinoic acid (ATRA, **3**), an endogenous ligand of retinoic acid receptors (RARs).[11] CRABP-I is thought to be related to metabolism of retinoic acid (RA) and resistance to RA in cancer cells,[12] while CRABP-II is suggested to be associated with nuclear transportation of RA.[13] It has also been reported that

(5) A non-peptide protac, utilizing murine double minute 2 (MDM2) as an E3 ligase and a non-peptide ligand (nutlin-3) as a MDM2 ligand, degraded androgen receptor (AR) (ref 4c). However, MDM2 naturally induces ubiquitination of AR when Nutlin-3 binds to MDM2. Gaughan, L.; Logan, I. R.; Neal, D. E.; Robson, C. N. *Nucleic Acids Res.* **2005**, *33*, 13–26. Logan, I. R.; McNeill, H. V.; Cook, S.; Lu, X.; Lunec, J.; Robson, C. N. *Prostate* **2007**, *67*, 900–906. Therefore, additional data to compare AR levels with nutlin-3 treatment and protac treatment would be needed to determine whether the decrease of AR is associated with the nutlin-3 derivative or the protac.

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CRABP-I is related to Alzheimer's disease[14] and CRABP-II is associated with neuroblastoma,[15] Wilms tumor,[15] and head and neck squamous cell carcinoma (HNSCC).[16] Therefore, CRABPs could be target proteins for treatment of these diseases. However, compounds which directly control the function(s) of CRABPs have never been reported, and the biological/physiological roles of these proteins remain unclear. Thus, small molecules that suppress the function of CRABPs would be of great interest, not only as tools for probing the biological/physiological roles of CRABPs but also as potential therapeutic agents for Alzheimer's disease and cancer.

Herein, we describe a protein knockdown approach targeting CRABPs focusing on (i) design and synthesis of hybrid molecules consisting of MeBS (**2**) and ATRA (**3**) connected via a spacer, (ii) CRABPs-degrading activity of the hybrid molecules, (iii) the mechanism through which CRABP-degradation is induced by the hybrid molecules, and (iv) inhibitory activity of the hybrid molecules on tumor invasion in cell-based assay.

Results and Discussion

Molecular Design. We speculated that the E3 ligase activity of cIAP1 might be harnessed for targeted protein degradation. In other words, if cIAP1 and a target protein could be induced to form an artificial (nonphysiological) complex, the target protein should be ubiquitinated by cIAP1, as cIAP1 ubiquitinates proteins that are bound to it. Thus, we hypothesized that hybrid small molecules, i.e., conjugates of ligands for target proteins with membrane-permeable MeBS (**2**), might induce selective degradation of the target proteins (Scheme 1c). Such hybrid small molecules were expected to strictly mimic the cognate complex for ubiquitination and to have sufficient membrane permeability for practical application.

To test this idea, we designed the hybrid molecules **4**, consisting of MeBS (**2**) coupled via a spacer moiety to ATRA (**3**), the specific ligand of CRABPs. The spacer was linked to the ester position of MeBS (**2**) and to the C4 position of ATRA (**3**). These positions were selected on the basis of our previous studies showing that (i) introduction of a bulky substituent at the ester moiety of MeBS (**2**) does not affect the binding affinity for cIAP1,[10b] (ii) introduction of a substituent at the C4 position of ATRA (**3**) does not affect the binding affinity for CRABP,[17] and (iii) the latter modification causes loss of ATRA's binding affinity for RARs (see our previous report and X-ray structure in the Supporting Information, Figure S1).[18] Observations (ii) and (iii) suggest that **4** would selectively bind to CRABPs, but not to RARs. Since the length of the spacer is likely to influence the efficiency of ubiquitination of target proteins, we designed and synthesized three compounds **4a**−**c** (Figure 1) with different spacer lengths.

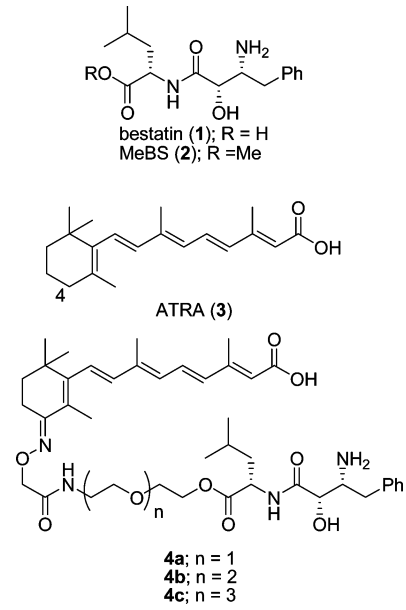

Figure 1. Structures of bestatin (**1**), MeBS (**2**), ATRA (**3**), and compounds **4**.

Synthesis. The designed compounds were synthesized as illustrated in Scheme 2. Compound **5** was prepared from ATRA (**3**) by esterification with 2-cyanoethyl alcohol, oxidation with excess MnO_2, and oximation with *O*-(carboxylmethyl)hydroxylamine.[18d,19] Compound **6** was prepared by protection of the amino group of bestatin (**1**). Condensation of compound **6** with alcohols **7a**−**c**[20] in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 1-hydroxybenzotriazole hydrate (HOBt·H_2O) afforded esters **8a**−**c**. Amines **9a**−**c** were obtained from compounds **8a**−**c** by removal of the Boc group under acidic conditions. Amidation of compound **5** and amines **9a**−**c** gave amides **10a**−**c** in good yield. Deprotection of the 9-fluorenylmethyloxycarbonyl (Fmoc) groups and 2-cyanoethyl groups of compounds **10a**−**c** with tetrabutylammonium fluoride (TBAF)[21] and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[22] gave compounds **4a**−**c**.

Decrease of CRABPs by the Synthesized Compounds. MOLT-4 cells express cIAP1 and CRABP-I (but not CRABP-II), and HT1080 cells express cIAP1 and CRABP-II (but not CRABP-I), as examined by Western blotting analysis (Supporting Information, Figure S2). Therefore, we examined the effects of **4** on the levels of CRABP-I in MOLT-4 cells and CRABP-II in HT1080 cells by Western blot analysis. As shown in Figure 2a, compounds **4** induced a dose-dependent decrease of CRABP-I protein in MOLT-4 cells. Compound **4b** seemed to be the most potent, and **4a** seemed to be the least potent among

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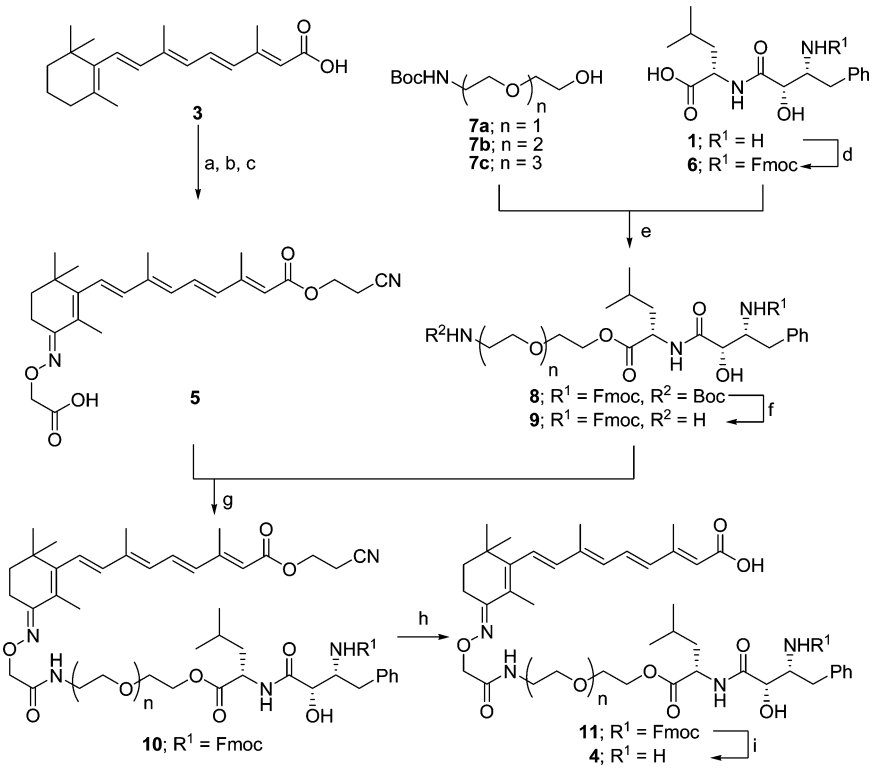
Scheme 2. Synthesis of Compounds **4**[a]



[a] Reagents and conditions: (a) 2-cyanoethyl alcohol, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), *N*,*N*-dimethylaminopyridine (DMAP), CH_2Cl_2, room temperature, 18 h, 90%; (b) MnO_2, CH_2Cl_2, room temperature, 22 h, 36%; (c) *O*-(carboxylmethyl)hydroxylamine, pyridine, room temperature, 17 h, 100%; (d) 9-fluorenylmethyloxycarbonyl chloride (FmocCl), K_2CO_3, THF, H_2O, room temperature, 24 h, 97%; (e) EDCI,1-hydroxybenzotriazole hydrate (HOBt·H_2O), *i*-Pr_2NEt, CH_2Cl_2, room temperature, 24 h, 14−51% (from **6**); (f) HCl, 1,4-dioxane, room temperature, 1 h, quant; (g) EDCI, HOBt·H_2O, Et_3N, CH_2Cl_2, room temperature, 14 h; (h) tetrabutylammonium fluoride (TBAF), MeOH, THF, room temperature, 1 h; (i) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), *n*-$C_{12}H_{25}SH$, CH_2Cl_2, room temperature, 3 h, 25−46% (three steps from **5**).

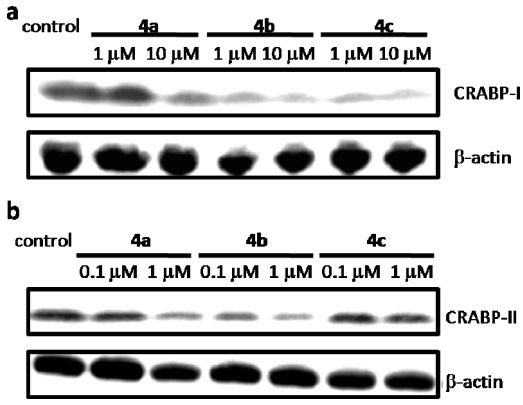


Figure 2. Down-regulation of CRABP-I and CRABP-II by treatment with compounds **4a**−**c**: (a) Western blot detection of CRABP-I levels in MOLT-4 cells after 16 h treatment; (b) Western blot detection of CRABP-II levels in HT1080 cells after 6 h treatment.

the three compounds, though the differences were not large. Compounds **4** also induced a dose-dependent decrease of CRABP-II in HT1080 cells. In this case, **4a** and **4b** strongly reduced the level of CRABP-II (**4b** seemed to be more potent than **4a**), while **4c** was relatively ineffective (Figure 2b). These results suggested that the linker with $n = 2$ is preferable among those investigated. A possible interpretation of this result would be that the structure of compound **4b** is most suitable to bring the ubiquitination sites in CRABPs and the RING domain of cIAP1 into an appropriate spatial relationship for effective ubiquitination to occur. The difference in CRABP-I/II selectivity of **4a** and **4c** suggests that the longer linker is better for down-regulation of CRABP-I, whereas the shorter linker is better for down-regulation of CRABP-II. The differences between CRABP-I and CRABP-II responses to treatment with **4a** and **4c** presumably reflect the structural differences between CRABP-I and -II.

Mechanism of Decrease in CRABPs. We next investigated the mechanism by which CRABPs are downregulated. First, we prepared HT1080 cells expressing FLAG-tagged cIAP1 and examined the influence of **4** on cIAP1 level by Western blot analysis, since esterified analogues of bestatin (**1**) induce autoubiquitination of cIAP1. All the compounds **4a**−**c** showed a dose-dependent cIAP1-decreasing effect, but their efficacy was lower than that of MeBS (**2**) (Figure 3a, Supporting Information, Figure S3). The reason for the weaker activity of **4** compared with MeBS (**2**) is not clear, but it might be due to the bulky ester group or to competing ubiquitination activity toward CRABP-II and cIAP1 itself. A decrease in cIAP1 level was also seen in MOLT-4 cells (Supporting Information, Figure S4).

Next, we pretreated the cells with an excess amount of MeBS (**2**) to investigate whether the reduction of CRABP-II by **4** was mediated by cIAP1 (Figure 3b). The pretreatment with an excess amount of MeBS (**2**) resulted in complete disappearance of cIAP1 but did not influence the CRABP-II level (lane 2). Both CRABP-II and cIAP1 levels were decreased by treatment with **4b** [without pretreatment with MeBS (**2**)] as mentioned above (lane 3). On the other hand, compound **4b** had no effect on CRABP-II levels in cells pretreated with 1000 μM MeBS (**2**) (lane 4). These results indicate that **4b** reduces the CRABP-II

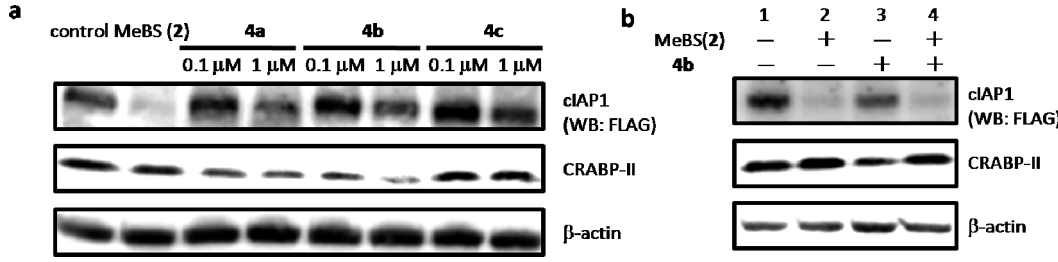


Figure 3. Western blot detection of CRABP-II and cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1: (a) CRABP-II and cIAP1 levels after 6 h treatment with **4**; (b) influence of pretreatment of MeBS (**2**) on CRABP-II degradation induction. The cells were treated with 1 μM **4b** for 3 h. MeBS (**2**) (1000 μM) was added to the culture 1 h prior to the addition of **4b**.

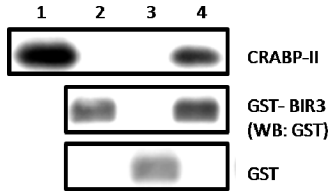


Figure 4. Western blot detection of GST-BIR3, GST, and CRABP-II levels of samples prepared by pull-down assay in vitro: lane 1, CRABP-II levels; lane 2, mixture of GST-BIR3 and CRABP-II; lane 3, mixture of GST, CRABP-II, and **4b**; lane 4, mixture of GST-BIR3, CRABP-II, and **4b**.

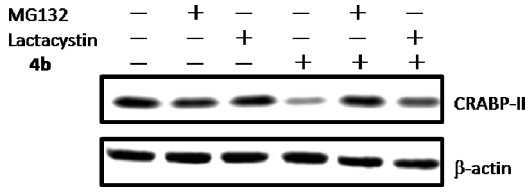


Figure 5. Influence of pretreatment with proteasome inhibitors on CRABP-II degradation induction. Western blot detection of CRABP-II and cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1. The cells were treated with 1 μM **4b** for 6 h. MG132 (10 μM) and lactacystin (10 μg/mL) were added to the culture 30 min prior to the addition of **4b**.

level in cIAP1-expressing cells but not in cIAP1-depleted cells, suggesting that cIAP1 mediates the ubiquitination of CRABP-II protein for proteasomal degradation in these cells.

We next examined the formation of a ternary complex consisting of **4b**, cIAP1, and CRABP-II by means of GST pull-down assay using the GST-tagged BIR3 domain of cIAP1, to which MeBS (**2**) binds. As shown in Figure 4, GST-BIR3 coprecipitated CRABP-II in the presence of **4b** (lane 4) but not in the absence of **4b** (lane 2). GST did not pull down CRABP-II even in the presence of **4b** (lane 3). This result indicates that CRABP-II is held in proximity to cIAP1 by **4b**, as we had hoped.

Next, we investigated the influence of proteasome inhibitors on the CRABP-II level in **4b**-treated cells. (Figure 5). The reduction of CRABP-II by **4b** was blocked by proteasome inhibitors MG132 and lactacystin. Thus, the decrease of CRABP-II induced by **4b** can be attributed to proteasomal degradation.

In addition, we confirmed that the decrease in CRABPs was not caused by a partial structure of **4** or by a mere mixture of MeBS (**2**) and ATRA (**3**) (Figure 6). Compound **4b** induced a decrease of CRABP-II, whereas the mixture of MeBS (**2**) and ATRA (**3**) did not. The treatments with 1 μM MeBS (**2**), and the combination of 1 μM MeBS (**2**) and 1 μM ATRA (**3**) decreased the cIAP1 level but did not affect the CRABPs level in HT1080. ATRA (**3**) at 1 μM did not cause any decrease of cIAP1 or CRABP. Similar results were obtained when HT1080

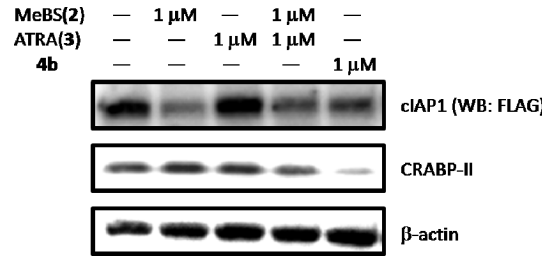


Figure 6. Influence of combination of MeBS (**2**) and ATRA (**3**). Western blot detection of CRABP-II and cIAP1 levels in HT1080 cells expressing FLAG-tagged cIAP1. The cells were treated with 1 μM MeBS (**2**), 1 μM ATRA (**3**), or 1 μM **4b** for 6 h.

or MOLT-4 cells were treated with 10 μM MeBS (**2**) and ATRA (**3**) (Supporting Information, Figure S5). Thus, conjunction of MeBS (**2**) and ATRA (**3**) within a single molecule is essential for CRABP degradation-inducing activity.

It was reported that ATRA (**3**) influences CRABPs levels by transactivation of RAR[23] or that ATRA (**3**) shows effects on HL-60 cell differentiation.[24] However, **4b** did not show agonistic activity toward RARs in reporter gene assay (Supporting Information, Figure S6). Compound **4b** did not show effects on HL-60 cell differentiation or did not enhance cell differentiation induced by ATRA (**3**) (data not shown). Addtionally, **4b** did not induce degradation of RARα (Supporting Information, Figure S7) and did not bind to RARs (Supporting Information, Figure S8). Thus, **4b** showed selective degradation-inducing activity for CRABPs, but not RARα or β-actin, as judged from Western blot experiments with equal loadings of total protein. These results indicated that **4b** has high specificity for degradation of CRABPs. All the results are consistent with our hypothesis that the hybrid molecules **4** form an artificial ternary complex with cIAP1 and CRABP, in which cIAP1 ubiquinates CRABP, leading to its degradation by proteasome.

These small molecules **4** have sufficient membrane permeability and stability to be used in cell systems and are effective in low concentration. Therefore, they might be suitable for a variety of studies in cells and/or animals. In addition, this protein knockdown strategy might be generally adaptable to a wide range of proteins by replacing ATRA (**3**) with a specific ligand for the target protein. Therefore, protein knockdown could be a simple and easy technique to complement genetic ablation at the DNA level (gene knockout) or the mRNA level (gene knockdown).

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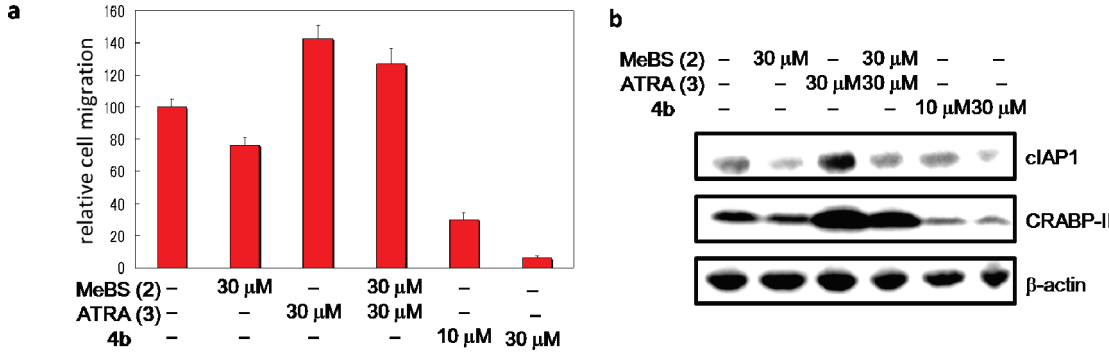

Figure 7. Suppression of cell migration by compound **4b** and degradation of CRABP-II in IMR-32 cells. (a) Relative IMR-32 cell migration. Results are presented as means ± SEM (performed in triplicate). (b) Western blot detection of CRABP-II and cIAP1 levels in IMR-32 cells after 24 h treatment with each reagent.

Inhibitory Activity on Tumor Invasion in Cell-Based Assay. Finally, we tested the effect of **4b** on neuroblastoma cells to explore the potential clinical usefulness of CRABP-II degradation inducers. It has been reported that CRABP-II is closely associated with development of neuroblastoma, Wilms tumor and HNSCC, and a reduction of CRABP-II level suppresses the migration of tumor cells.[15,16] Therefore, we evaluated the migration-suppressing activity of **4b** toward human neuroblastoma IMR-32 cells (Figure 7). Treatment of the IMR-32 cells with 30 μM MeBS (**2**) slightly reduced the cell migration by approximately 30%, in agreement with previous reports that MeBS (**2**) shows antitumor activity.[10a,25] Interestingly, treatment of the cells with ATRA (**3**) alone or the combination of MeBS (**2**) and ATRA (**3**) enhanced the cell migration by 30−40%, possibly as a result of up-regulation of CRABP-II expression by ATRA (**3**). As expected, treatment of the cells with 10 and 30 μM **4b** remarkably reduced the cell migration by approximately 75% and 95%, respectively. Moreover, the extent of migration inhibition was well correlated with the CRABP-II level in the cells. MeBS (**2**) did not affect CRABP-II levels in IMR-32 cells, while ATRA (**3**) or the combination of MeBS (**2**) and ATRA (**3**) up-regulated CRABP-II levels. Since compound **4b** induced degradation of CRABP-II, it might be a useful tool for studying the function(s) of CRABP-II.

Targeted cancer therapy blocks the growth of cancer cells by interfering with specific molecules required for carcinogenesis and tumor growth, rather than by simply interfering with rapid cell division, as is done with traditional chemotherapy.[26] Targeted cancer therapies may be more effective than traditional chemotherapy and less harmful to normal cells. Many kinds of target proteins related to cancer have been identified so far. Further, small-molecular inhibitors for target enzymes or modulators for target receptors have been discovered, and some of them are used for targeted cancer therapy. But many cancer-related proteins, including CRABP-II, can not currently be functionally regulated with small molecules. CRABP-II is expressed in several cancers, including breast cancer,[27] Wilms tumor,[28] HNSCC,[16] ovarian cancer,[29] gastric cancer,[30] uterine leiomyoma,[31] and melphalan- or phorbol-ester-resistant cell lines,[32] suggesting that it may play a role in cancer development. In Wilms tumor, CRABP-II overexpression has been reported to correlate with poor clinical outcome.[15] Here, we found that the small-molecular CRABP-II degradation inducer **4b** inhibited migration of neuroblastoma cells. Thus, CRABP-II degradation inducers may be effective for therapy of neuroblastoma and other CRABP-II-overexpressing cancers. Further studies on various types of cancer cells, including cell proliferation assay, are in progress.

The strategy described in this paper might also be adaptable to a range of cancer-related proteins by replacing ATRA (**3**) with specific ligands for the target proteins. In addition, suppression of cIAP1 function is thought to be favorable for cancer treatment,[33] and disruption of the cIAP1 gene in mice results in no obvious abnormality.[34] Therefore, suppression or degradation of cIAP1, which is overexpressed in several human cancers,[7] should not vitiate the anticancer effect. On the other hand, protacs have utilized peptides recognized by two kinds of E3 ligase complex, von Hippel−Lindau tumor suppressor (VHL), which is deficient or mutated in several cancer,[35] and

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β-transducin repeat-containing protein (β-TRCP) Skp1-Cullin-F box (SCF) complex ($SCF^{\beta\text{-TRCP}}$), which plays important roles in many tissues.[36] Those peptide-based protacs, especially those utilizing $SCF^{\beta\text{-TRCP}}$,[4a,b] seem best suited to cell-free systems and might be difficult to use in cancer treatment due to stability issues. Evaluation of our protein degradation inducers, MeBS (**2**)−ligand hybrid molecules, for targeted cancer therapy seems warranted.

Conclusion

We designed and synthesized CRABPs degradation inducers **4**, in which a ligand for CRABPs is conjugated with MeBS (**2**) via a spacer, aiming to make use of the ubiquitin E3 ligase activity of cIAP1. Compounds **4** induced down-regulation of CRABPs in cells. Our results indicate that these small molecules induce the formation of an artificial (nonphysiological) ternary complex of cIAP1 and CRABPs, leading to degradation of CRABPs via the ubiquitin-proteasome pathway. Compound **4b** has sufficient activity, permeability, and stability for use in cellular systems, and we confirmed that it inhibited migration of neuroblastoma IMR-32 cells.

This strategy for protein knockdown should be widely applicable by replacing ATRA (**3**) with specific ligands for other target proteins and should provide a methodology to complement gene knockout and gene knockdown techniques. It is expected to be useful for probing biological functions of proteins and might also be applicable for targeted cancer therapy using MeBS (**2**) conjugated with ligands for cancer-related proteins.

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Supporting Information Available: Experimental procedure, copies of ^{1}H NMR spectra, Figures S1−S8, and complete ref 35c. This material is available free of charge via the Internet at http://pubs.acs.org.

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